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Bli et al., Afr J Tradit Complement Altern Med. (2017) 14(2):47-59
doi:10.21010/ajtcam.v14i2.6

EFFICACY AND SAFETY OF BAN HUANG ORAL LIQUID FOR TREATING BOVINE RESPIRATORY DISEASES

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Abstract

Background: Ban Huang oral liquid was developed as a veterinary compound preparation by the Lanzhou Institute of Husbandry and Pharmaceutical Sciences of the Chinese Academy of Agricultural Sciences (CAAS). The purpose of this study was to determine whether the oral liquid preparation of traditional Chinese medicine, Ban Huang, is safe and effective for treating respiratory diseases in cattle.

Materials and Methods: Acute oral toxicity experiments were conducted in Wistar rats and Kunming mice via oral administration. The minimum inhibitory concentration of the drug against *Mycoplasma bovis in vitro* with the double dilution method was 500 mg/mL, indicating good sensitivity. The results of laboratory pathogen testing, analysis of clinical symptoms, and analysis of pathological anatomy were combined to diagnose bovine respiratory diseases in 147 Simmental cattle caused by mixed infections of *M. bovis*, bovine respiratory syncytial virus, bovine parainfluenza virus type 3, and *Mannheimia haemolytica*. These cattle were randomly divided into three groups: drug treatment group 1 (treated via Tilmicosin injection), drug treatment group 2 (treated with Shuang Huang Lian oral liquid combined with Tilmicosin injection), and drug treatment group 3 (treated with Ban Huang oral liquid combined with Tilmicosin injection). Treatment effects were observed within 7 days.

Results: The results showed no toxicity and a maximum tolerated dose greater than 20 g/kg BW. For the 87 cattle in drug-treatment group, the cure rate was 90.80%, whereas the response rate was 94.25%. The cure rate of drug treatment group was increased by 14.13% in comparison with that of drug control group 1 and by 7.47% in comparison with that of drug control group 2 (both $P < 0.05$).

Conclusion: This study demonstrates that Ban Huang oral liquid is a safe and effective treatment for bovine respiratory diseases, especially for mixed infection caused by *M. bovis*, bacteria, and viruses.

Key words: Ban Huang oral liquid, *Mycoplasma bovis*, minimum inhibitory concentration, safety, clinical efficacy

Introduction

Bovine respiratory diseases threaten the healthy development of the cattle breeding industry throughout the world. Ranking second only to severe infectious diseases in incidence and mortality, respiratory disease is the most significant and widespread cause of economic loss in the beef cattle industry (Ellis, 2001). The Chinese Ministry of Agriculture has organized relevant experts to conduct in-depth investigations on recent prevalent bovine respiratory diseases in

China. By combining laboratory pathogen testing with analysis of clinical symptoms and epidemiological surveys, it was found that the main pathogen inducing respiratory diseases in cattle in China was *Mycoplasma bovis*, followed by *Pasteurella multocida*, bovine respiratory syncytial virus, and bovine parainfluenza virus type 3 (BPIV-3), all of which can join in mixed infections. The *M. bovis* pathogen is a particularly serious concern for cattle breeders because of the relative difficulty in diagnosing respiratory disease caused by the mycoplasma, the lack of an effective antibiotic against it, and its persistence and transmissibility (Caswell, 2007; Fox et al., 2005). The first report on the connection between *M. bovis* and bovine respiratory diseases was published in 1976 (Nicholas and Ayling, 2003). Although *M. bovis* can act as a primary pathogen, many cases of disease showed co-infection with other bacteria or viruses (Caswell et al., 2010).

The worldwide spread of *M. bovis* has recently gained momentum (Cai et al., 2005; van der Burgt et al., 2008; Dyer et al., 2008), causing significant economic losses for the cattle industry. For example, the annual economic loss in the United States due to bovine respiratory diseases reached 500 million US dollars (Wilkinson, 2009; Miles, 2009). In Holland, Laak et al. found that the rate of *M. bovis* infection was greater than 20% at a cattle-fattening farm, where few cattle were considered completely healthy (Buchvarova and Vesselinova, 1989). In Switzerland, bovine respiratory diseases caused by *M. bovis* account for more than 50% of all respiratory diseases and reduce the growth rate of latently infected herds by 8% (Poumarat et al., 2001). Nearly all outbreaks of *M. bovis* infection in China are related to transport, with most cattle showing signs of onset approximately one week after arriving at their destination (Shi et al., 2008). To date, there is no effective vaccine to prevent *M. bovis* infection (Mulongo et al., 2013). Currently, bovine respiratory disease associated with *M. bovis* infection is treated primarily with antibiotics. However, antibiotic therapy is rarely effective, the prevalence of antimicrobial resistance is reportedly increasing (Gautier-Bouchardon et al., 2014; Li et al., 2011), and antibiotic residue in cattle poses a serious threat to food safety and public health.

Traditional Chinese medicine (TCM) has played an important role in health protection and disease control for millennia. The therapeutic efficacy of TCM is based on the combined actions of constituents of phytochemical mixtures. TCM is utilized in many countries and districts, and the global market for herbal medicine is growing annually. TCM is widely accepted in Asian countries, and its popularity is also increasing in western countries. For example, the publication "Guidance for Industry: Botanical Drug Products" produced by the US Food and Drug Administration signals basic acceptance of TCM in western countries (Xu, 2008). TCM is useful in the prevention and control of bacterial infections (Wei et al., 2008). Huang et al. (Huang et al., 2005) generated a TCM preparation using the extracts of ten medicinal herbs (including *Chrysanthemum indicum*, *Andrographis paniculata*, Radix bupleuri, *Houttuynia cordata*, and *Folium isatidis*) and tested its protective effects against avian influenza virus H9N2, Newcastle disease virus, infectious bronchitis virus, and *Mycoplasma gallisepticum* using challenge experiments, demonstrating that the TCM preparation effectively controlled respiratory disease in poultry caused by these four pathogens. Qingkailing Injection was developed based on the ingredients in the Cow-Bezoar Bolus for Resurrection, the primary TCM preparation used to treat febrile and epidemic diseases. Qingkailing Injection is an outstanding representative of heat-clearing and detoxifying Chinese patent medicines that promotes regaining consciousness and has antibacterial, antiviral, antipyretic, and analgesic actions. Qingkailing Injection contributed greatly to efforts to control SARS, influenza A virus subtype H1N1, human avian influenza, and hand-foot-mouth disease (Guo, 2015; Lu et al., 2006). Zhang et al. (Zhang et al., 2007) controlled nephropathogenic infectious bronchitis in broiler chickens by adding veterinary Hushensuxiaosan Decoction (Shaanxi ShengAo Animal Pharmaceutical Co., Ltd.) and Shenzhongbaidu Decoction (Jiangxi Zhongcheng Medicine Group Co., Ltd.) to a centralized drinking water system. Studies have demonstrated that Chinese medicinal herbs can fully activate the immune system to enhance immunity. Chinese medicinal herbs have the advantages of easy accessibility, low cost, low toxicity, and drug tolerance (Huo and Li, 2002; Shen et al., 2004; Zhang et al., 1998).

The goal of this study was to prepare a compounded Chinese medicinal prescription to treat bovine respiratory diseases, especially pneumonia, caused by mycoplasmic infections. TCM drugs can be used to reduce antibiotic tolerance and residue in cattle. Ban Huang oral liquid was developed as a veterinary compound preparation by the Lanzhou Institute of Husbandry and Pharmaceutical Sciences of Chinese Academy of Agricultural Sciences (CAAS). The main ingredients of Ban Huang oral liquid included *Isatidis Radix*, *Coptidis Rhizoma*, *Lonicerae Joponicae Flos*, *Scutellariae Radix*, *Anemarrhenae Rhizoma*, *Glycyrrhizae Radix et Rhizoma*.*Isatidis Radix*, *Coptidis Rhizoma*, *Lonicerae Joponicae Flos*, and *Scutellariae Radix*, which have heat-clearing and detoxifying properties, as well as blood-cooling, throat-easing, and antibacterial actions (Li, 2005; Chinese Pharmacopoeia Commission, 2010). *Anemarrhenae Rhizoma* has heat-clearing, fire-purging, yin-nourishing, dryness-moistening, blood-cooling,

throat-easing, and anti-inflammatory actions (Chinese Pharmacopoeia Commission, 2010). *Glycyrrhizae Radix et Rhizoma* has the effects of invigorating the spleen, replenishing qi, heat-clearing, detoxifying, and expelling phlegm to arrest coughing; therefore, it is used for the coordination of various drugs (Chinese Pharmacopoeia Commission, 2010).

The combined use of the ingredients in Ban Huang oral liquid can achieve the effects of heat-clearing, detoxifying, removing dampness and swelling, arresting bleeding, and easing throat soreness; indeed, these ingredients have been applied to treat many febrile diseases. *Coptidis Rhizoma* and *Scutellariae Radix*, the main ingredients in Ban Huang oral liquid intended to treat respiratory diseases, have heat-clearing, detoxifying, and antibacterial actions. Berberine hydrochloride and baicalin were chosen as indices for quality control of *Coptidis Rhizoma* and *Scutellariae Radix*, respectively, because the detection methods for these compounds are stable and reliable.

Shuanghuanglian oral liquid (composed of *Lonicerae Joponicae Flos*, *Scutellariae Radix*, and *Forsythiae Fructus*) (Jia, 2013) was compared with Ban Huang oral liquid because Shuanghuanglian oral liquid the former has heat-clearing, detoxifying, and antibacterial actions. It is widely used in the clinical mainly for to treat respiratory infections, including pneumonia., etc. (Jia, 2013; Zhang, 2010). Through the acute toxicity experiments and clinical pharmacodynamic studies presented here, this study demonstrates that Ban Huang oral liquid is a safe and effective treatment for bovine respiratory diseases, especially mixed infection caused by *M. bovis*, bacteria, and viruses.

Materials and Methods

Materials

Isatidis Radix, *Coptidis Rhizoma*, *Lonicerae Joponicae Flos*, *Scutellariae Radix*, *Sophorae Tonkinensis Radix et Rhizoma*, *Arctii Fructus*, *Platycodonis Radix*, *Anemarrhenae Rhizoma*, and *Glycyrrhizae Radix et Rhizoma* were purchased from the Lanzhou HuiRenTang Pharmacy and certified by GSP. Ban Huang oral liquid (1 g/mL crude drug, batch no. 20090615) was provided by the Lanzhou Institute of Husbandry and Pharmaceutical Sciences, CAAS.

Shuanghuanglian oral liquid (1 g/mL crude drug, batch no. 2006 020115030) was provided by Tianjin Zhongsheng TiaoZhan Bioengineering Co., Ltd. Tilmicosin injection (3 g/10mL, batch no. 20090717) was provided by Sichuan Zhibang Biological Technology Co., Ltd. Baicalin (batch no. 110715-200815) and berberine hydrochloride (batch No. 110713-200911) were provided by the National Institute for the Control of Pharmaceutical and Biological Products and used as standards. Chromatographically pure acetonitrile and methanol were obtained from Fisher Company (USA). Industrial grade ethanol, 95% ethanol, potassium dihydrogen phosphate, sodium dodecyl sulfate, sodium benzoate, phosphoric acid, agar, thallium acetate, penicillin, sodium hydroxide, and phenolsulfonphthalein were obtained from Sinopharm Group Co. Ltd. PPLO broth base was obtained from BD Co. (USA). Glucose and β-nicotinamide adenine dinucleotide were obtained from Sangon Biotech (Shanghai) Co., Ltd. Fetal bovine serum was obtained from Zhejiang Tianhang Biological Technology Co., Ltd. *M. bovis* strain gs-1 was isolated, identified, and preserved by the Lanzhou Institute of Husbandry and Pharmaceutical Sciences, CAAS.

Equipment

A Waters 2695 HPLC System coupled with a UV detector (Waters Co., USA), an AEL-1600 electronic balance (Shimadzu, Japan), and a Millipore water purification system (USA) were used in these experiments.

Ethics statement

The protocol was approved by the Ethics Committee of Animal Experiments of Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS, which was commissioned by The International Cooperation Committee of Animal Welfare (ICCAW) affiliated with the China Association for the Promotion of International Agricultural Cooperation (CAPIAC). The mouse toxicity study and cattle treatment procedures were subject to the ethical oversight of the Ethics Committee of Animal Experiments of Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS and implemented in accordance with the technical guidelines for clinical trials for veterinary TCM and natural drugs.

doi:10.21010/ajtcam.v14i2.6

Study protocol

This study used six batches of Ban Huang oral liquid prepared with stable and quality controllable processes. Quality control indices were detected using high performance liquid chromatography (HPLC). Inter-batch differences were tested; relative standard deviation (RSD) was required to be less than 5%. Acute oral toxicity experiments were conducted in rats to evaluate the clinical safety of the drug. The sensitivity of *M. bovis* to the drug was determined according to the guidelines for therapeutic drug trials in the Collections of Technical Requirements for Veterinary Drug Trials (Ministry of Agriculture, 2001). The method for diagnosing bovine diseases was established along with the criteria for evaluating the treatment effect. The mortality, cure rate, and response rate were calculated after Ban Huang oral liquid was administered. The results were compared with those obtained using Shuang Hang Lian oral liquid.

Intergroup differences were statistically tested, and the efficacy of Ban Huang oral liquid for treating bovine respiratory infectious diseases was evaluated.

Preparation of Ban Huang oral liquid

The optimal soaking time was determined based on the water absorption of the drug using the concentration of the quality control ingredients as indicators. The water extraction process was optimized using L_9 (3^4) orthogonal array testing. An analysis of variance was performed to determine and verify the process. The concentration and relative density were measured, and the clarity and transport rate of the main quality control ingredients were used as indicators to characterize the alcohol precipitation method.

Concentration determination for quality control

An HPLC-UV method was used to determine the concentrations of baicalin and berberine hydrochloride in Ban Huang oral liquid. The baicalin reference and berberine hydrochloride reference were accurately weighed, after which reference solutions of 5 mg/mL and 10 mg/mL, respectively, were generated using methanol. Six batches of Ban Huang oral liquid (1 mL each) were individually weighed and placed into separate 50-mL flasks. The solution was diluted to 50 mL with methanol and mixed well. A C18 HPLC column was used with an aqueous solution of methanol and 0.1% phosphoric acid (50:50) as the mobile phase. Baicalin was detected at a wavelength of 274 nm (1.0 mL/min flow rate, 10 µL sample size). The number of theoretical plates was calculated using the baicalin peak and was required to be greater than 2,500. Octadecyl silane chemically bonded to silica was used as filler, and an aqueous solution of acetonitrile and potassium dihydrogen phosphate (47:53, v/v, 3.2 g potassium dihydrogen phosphate and 1.6 g sodium dodecyl sulfate in every 1 L of water) was used as the mobile phase. The concentration of berberine hydrochloride was detected at a wavelength of 347 nm (10 µL sample size). The number of theoretical plates was calculated using the berberine hydrochloride peak and was required to be greater than 5,000.

Drug safety evaluation in acute oral toxicity experiments in rats and mice

The protocol followed the guidelines recommended in the *Guidance of Veterinary Drugs for Technical Study*, 2006–2011 (Ministry of Agriculture, 2012). Sixty Kunming mice (30 female and 30 male) weighing 18–22 g (license no. scxk [Gansu] 2012-0075) and sixty Wistar rats (30 female and 30 male) weighing 200–250 g (license No. scxk [Gansu] 2012-0075) were used in the experiments. All rodents were purchased from the Medical School of Lanzhou University. The rats and mice were randomly divided into six groups (n = 10 per group, 5 males and 5 females) based on body weight. Intragastric irrigation was performed once daily. For control mice, normal saline was gavaged at a dose of 10 g/kg BW. Five experimental groups were gavaged at doses of 5, 10, 15, 18, or 20 g/kg BW (one dose per group). The mental state, behavior, and food intake of the mice were observed for 14 consecutive days.

Susceptibility testing

A PPLO broth base was supplemented with fetal bovine serum, 25% yeast leachate, 10% glucose, 10% arginine, 0.4% phenolsulfonphthalein, 1% thallium acetate, and 1% penicillin. The *M. bovis* strain was aspirated using a

micropipettor, inoculated into the PPLO broth, and cultured at 37 °C in a 5% CO_2 incubator for 48–72 h. The PPLO broth was added to 12 sterile screw-cap test tubes (1.8 mL each) using the double dilution method and cultured at 37 °C in a 5% CO_2 incubator for 48–72 h. The color change of the culture media was observed, and the color-changing unit (CCU) was determined as the concentration of the highest dilution that changed the color of the media. For example, if the 5th tube in the dilution series was the highest dilution to show a color change, then the CCU was 1×10^{-5}/mL.

The MIC of Ban Huang oral liquid was determined using the microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI/NCCLS, 2003). The optimal concentration of *M. bovis* in the drug sensitivity test was 1×10^{-3} to 1×10^{-4} CCU per 0.2 mL; a concentration of 1×10^{-4} CCU was used for MIC determination. The MIC was defined as the drug concentration in the tube before the first color change after 48–72 h of incubation at 37 °C. The experiment was repeated for confirmation when no tubes showed a color change after 3–6 d.

Study animals

A total of 147 Simmental cattle (aged 6–12 months) affected by respiratory diseases were reared in two-row barns at Fucheng Beef Cattle Farm in Sanhe City (Hebei Province) and Wanhe Beef Cattle Farm in Zhangye City (Gansu Province). All cattle were in the stage of disease onset and showed the following symptoms: increased body temperature (approximately 41°C), continued fever, poor appetite, coarse and disordered hair, emaciation, cough, shortness of breath, diarrhea, and hemafecia. We had the permission of the cattle farm owners to perform the treatment study.

Clinical diagnosis:

Diagnosis was made by combining the results of the clinical observations of symptoms with those of pathological anatomy analysis and laboratory pathogen testing. Laboratory pathogen testing was conducted as follows: a TaqVet Triplex *Pasteurella multocida*/*Mannheimia haemolytica* fluorescence qPCR kit and TaqVet *Mycoplasma bovis* fluorescence qPCR kits were used to examine mucus from the nasal cavities of affected cattle. DNA extraction was performed using a QIAamp DNA mini kit (QIAGEN, 51304). Bovine respiratory syncytial virus and BPIV-3 were detected using the TaqVet Triplex bRSV & PI3 kit with mucus from the trachea and bronchus. A QIAGEN kit (QIAamp Viral RNA Mini Kit: 52904 or 52906) was used for RNA purification and identification. Detections were conducted before and after treatments.

Efficacy evaluation

Cattle were considered cured when all clinical symptoms of respiratory diseases were absent, appetite and mental state were restored to normal, and laboratory pathogen tests of respiratory secretions were negative. Cattle were considered responsive when clinical symptoms were markedly alleviated, with restoration of appetite and mental state. The treatment was considered ineffective when clinical symptoms of respiratory diseases were not alleviated or were aggravated, appetite declined or was completely lost, mental state was poor, and laboratory pathogen tests of respiratory secretions were positive.

Treatment

The 147 affected cattle (clinically diagnosed and experimentally confirmed) were randomly divided into three groups. Drug control group 1 was comprised of 30 cattle that were treated with Tilmicosin injection at a dose of 10mg/kg body weight (BW) by subcutaneous injection once daily for 1 d. Drug control group 2 was comprised of 30 cattle that were treated with Shuang Hang Lian oral liquid at a dose of 0.4 mL/kg BW twice daily for 7 d, followed by combined treatment with Shuang Hang Lian oral liquid (0.4 mL/kg BW) and Tilmicosin injection (10 mg/kg BW) by subcutaneous injection for 1 d. The experimental drug treatment group (n = 87 cattle) was treated with Ban Huang oral liquid (0.4 mL/kg BW) twice daily for 7 d, followed by combined treatment with Ban Huang oral liquid (0.4 mL/kg BW) and Tilmicosin injection (10 mg/kg BW) by subcutaneous injection once daily for 1 d.

Results

Preparation of Ban Huang oral liquid

The optimal preparation process for Ban Huang oral liquid was determined through preliminary trials. *Isatidis Radix*, *Coptidis Rhizoma*, *Lonicerae Joponicae Flos*, *Scutellariae Radix*, *Sophorae Tonkinensis Radix et Rhizoma*, *Arctii Fructus*, *Platycodonis Radix*, *Anemarrhenae Rhizoma*, and *Glycyrrhizae Radix et Rhizoma* were soaked in water

for 12 h and boiled twice for 1 h each time. For the first boiling, 7 times the volume of water was added, and for the second boiling, 5 times the volume of water was added. The resulting liquid was combined and filtered, after which the filtrate was condensed to a relative density of 1.18–1.25 (25 °C). Next, ethanol was slowly added until the proportion reached 60%; this solution was mixed well and left to stand for 24 h. The supernatant was collected after filtration to recover ethanol until there was no ethanol aroma left in the remaining filtrate. Next, ethanol was added again until the proportion reached 75%, after which the solution was mixed and left to stand for 24 h. The supernatant was collected by filtration and ethanol was recovered until no ethanol aroma remained. Sodium benzoate (3 g) was added to the resulting solution, which was diluted with water to a volume of 1000 mL, mixed well, sterilized by boiling, and bottled under sterilized conditions.

Concentration determination

The prescription consisted of the nine herbs listed above. Berberine hydrochloride and baicalin were detected using HPLC to evaluate the preparation process and perform quality control of active ingredients *Coptidis Rhizoma* and *Scutellariae Radix*, respectively. The results of these assays demonstrated that the RSD values for baicalin and berberine hydrochloride in all six batches of Ban Huang oral liquid were less than 5%, indicating good reliability and stability of the preparation process (Tables 1, 2 and Fig 1, 2).

Table 1: Baicalin concentration in Ban Huang oral liquid.

Batch no.	Mean content ± SD (μg/mL)	RSD (%)
20100122	1032.55 ± 5.37	
20100123	1103.14 ± 2.55	
20100124	983.18 ± 3.18	4.37
20090614	1036.55 ± 6.73	
20090615	1068.97 ± 3.21	
20090616	1100.72 ± 6.42	

Table 2: HPLC chromatograms of berberine hydrochloride in Ban Huang oral liquid.

Batch no.	Mean content ± SD (μg/mL)	RSD (%)
20100122	33.56 ± 1.12	
20100123	30.17 ± 2.03	4.05
20100124	32.67 ± 0.98	
20090614	32.11 ± 1.31	
20090615	31.98 ± 0.83	
20090616	33.80 ± 1.24	

doi:10.21010/ajtcam.v14i2.6

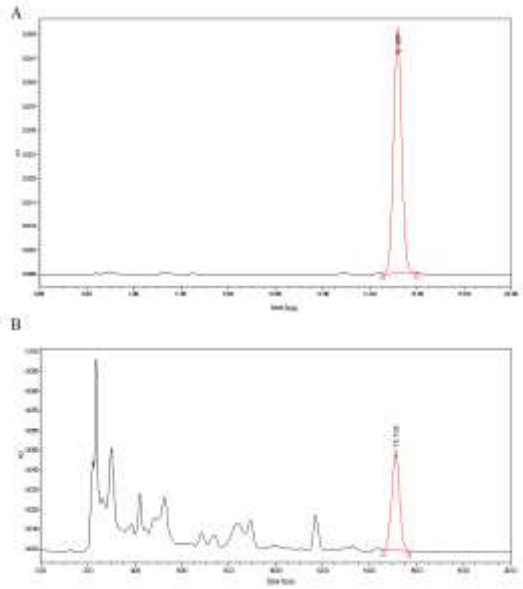

Figure1: Representative HPLC chromatograms showing baicalin peaks in Ban Huang oral liquid. (A) is the chromatogram of the reference substance, and (B) is the chromatogram of the sample.

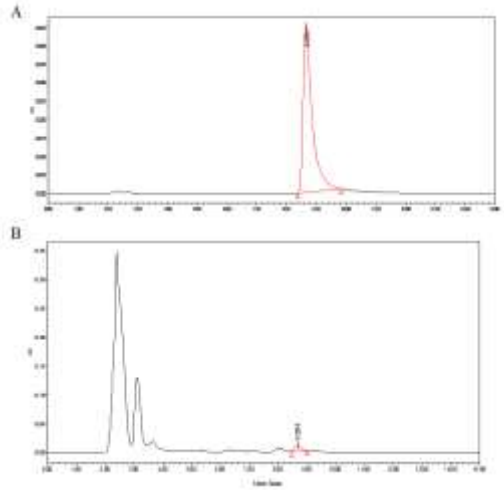

Figure 2: Representative HPLC chromatograms of berberine hydrochloride in Ban Huang oral liquid. (A) is the chromatogram of the reference substance, and (B) is the chromatogram of the sample.

Safety evaluation

Acute oral toxicity experiments were conducted in rats and mice (n = 10 of each in control and each treatment group). No rodents died following treatment with Ban Huang oral liquid (5, 10, 15, 18, or 20 mg/kg BW) for 14 days. During the treatment, rats and mice showed a normal mental state, normal food intake, and normal water intake, without any adverse reactions. The maximum tolerated dose (LD_0) of Ban Huang oral liquid in rodents was greater than 20 g/kg BW; thus, the median lethal dose (LD_{50}) is greater than this value. Therefore, according to the standards for toxicological classification of drugs, Ban Huang oral liquid was considered nontoxic.

No fatalities occurred in any of the groups of rats or mice, and no animal was sacrificed during this study.

doi:10.21010/ajtcam.v14i2.6

MIC determination

The *M. bovis* strain was cultured in an incubator for 48 h to 72 h until the color of the culture medium turned translucent yellow. Proliferating *M. bovis* cells were subjected to 10-fold serial dilution and cultured at 37 °C until the media did not change color after 48–72 h. The results demonstrated that the endpoint dilution was 1×10^{-7}. Thus, the CCU of *M. Bovis* strain gs-1 was 1×10^{7} CCU/mL. The MIC of Hainosankyuto was greater than 500 mg/mL (Fig. 3). These results indicate that Ban Huang oral liquid significantly inhibited visible growth of *M. bovis*.

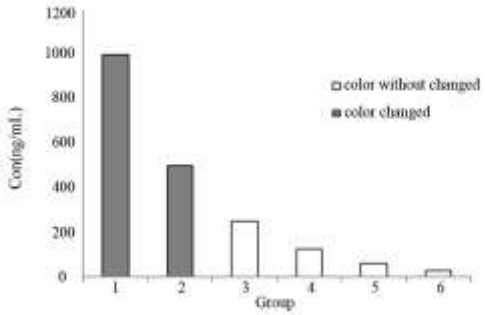

Figure 3: Inhibitory effect of Ban Huang oral liquid on the growth of *M. bovis* in vitro. The X-axis values (1–6) represent different concentrations of drug (1000 mg/mL, 500 mg/mL, 250 mg/mL, 125 mg/mL, 62.5 mg/mL, 31.25 mg/mL); 1 and 2, color changed; 3–6, no color changes; the lowest concentration of drug that inhibited visible growth of *M. bovis* was 500 mg/mL.

Diagnosis

The cattle showed clinical symptoms consistent with those of bovine respiratory disease. During early onset of respiratory disease, severely affected cattle stopped eating, while their body temperature increased to 41.3 ± 0.2 °C. The affected cattle had difficulty breathing, groaned, and presented with abdominal breathing and tenderness upon rib pressing. The cattle refused to crouch and demonstrated pain on coughing, which was aggravated early in the morning and during the night. Clear or purulent nasal discharge was observed with weakened or no vesicular breath sounds during lung auscultation. Death rattles and pleural friction sounds were heard. The stool was loose and contained blood in secondary diarrhea. Lameness and joint abscesses were observed along with secondary arthritis. Few severely affected cattle showed a poor mental state, disordered and lusterless hair, emaciation, or dry cough. In the late stage of infection, the affected cattle were severely anorectic and lay down; they exhibited shortness of breath or weak breathing and eventually died of respiratory failure.

As shown in Figure 4, the results of laboratory pathogen testing indicated that cattle infection was mainly caused by *M. bovis*. These results also demonstrated that Ban Huang oral liquid had the strongest inhibitory effect on *M. bovis* and the weakest inhibitory effect on BPIV-3, among the tested pathogens. After assessing the pathogen testing results with clinical symptoms, 147 cattle were diagnosed as having bovine respiratory diseases caused by *M. bovis*, bovine respiratory syncytial virus, *M. haemolytica*, and BPIV-3.

doi:10.21010/ajtcam.v14i2.6

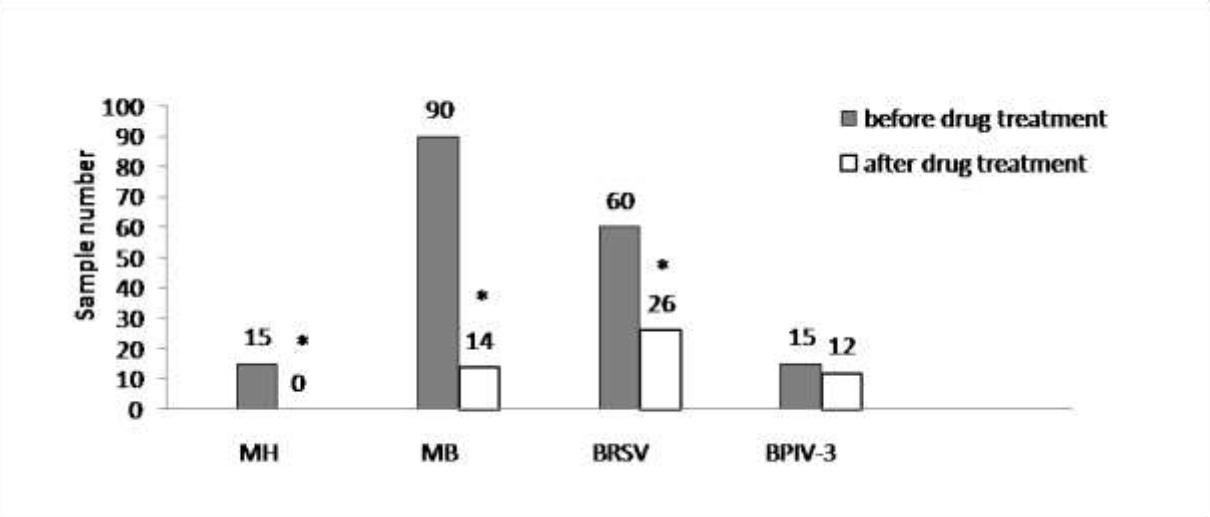

Figure 4: Results of laboratory pathogen testing in 147 cattle. 1: *Mannheimia haemolytica*; 2: *Mycoplasma bovis*; 3: bovine respiratory syncytial virus; 4: BPIV-3. Because *Pasteurella multocida* was not detected before or after drug treatment, results for this organism are not shown. *, significant difference.

Treatment outcome

After drug treatment for 7 days, clinical symptoms completely disappeared in some cattle, in which the results of laboratory testing for pathogens were negative; moreover, these cattle showed normal body temperature, uniform breathing without any coughing, and no death rattles or diarrhea. These cattle had a good mental state, glossy hair, and normal appetite; thus, they were considered cured. Some of the cattle tested positive for pathogens, but their symptoms were alleviated; these animals were considered responsive. Other animals showed no alleviation or even aggravation of symptoms; treatment was considered ineffective in these animals.

Variation in the body temperature of drug control group 1, drug control group 2, and the experimental drug treatment group over 7 days of treatment is shown in Fig. 5. The average temperature of drug control group 1 declined from 41.1 °C to 38.9 °C, a 1.2 °C reduction. The average temperature of drug control group 2 declined from 40.8 °C to 38.8 °C, a 2.0 °C reduction. For the experimental drug treatment group, the average body temperature declined from 41.1 °C to 38.6 °C, a 2.5 °C reduction. These results show that there was a significant decline in the body temperature of each group within 24 h after drug administration. Moreover, there was a significant difference in the decline in the body temperature of the experimental drug treatment group in comparison with that of drug control group 1 and drug control group 2 ($P < 0.05$).

After 7 days of treatment, 79 animals in the experimental drug treatment group were cured (90.80% cure rate), whereas 82 were responsive and 4 did not respond to the treatment, which was deemed ineffective. In drug control group 1, 23 animals were cured (76.67% cure rate) and 26 were responsive; the treatment was ineffective in 4 animals. In drug control group 2, 25 animals (83.33% cure rate) and 27 were responsive; the treatment was ineffective in 3 animals. These results are shown in Figure 6. No animal died in the experimental drug group, in which the cure rate was increased by 14.13% compared with that of drug control group 1 and by 7.47% compared with that of drug control group 2 (both $P < 0.05$). Thus, Ban Huang oral liquid was effective against bovine respiratory disease, superior to Shuang Hang Lian oral liquid and Tilmicosin alone, and nontoxic.

doi:10.21010/ajtcam.v14i2.6

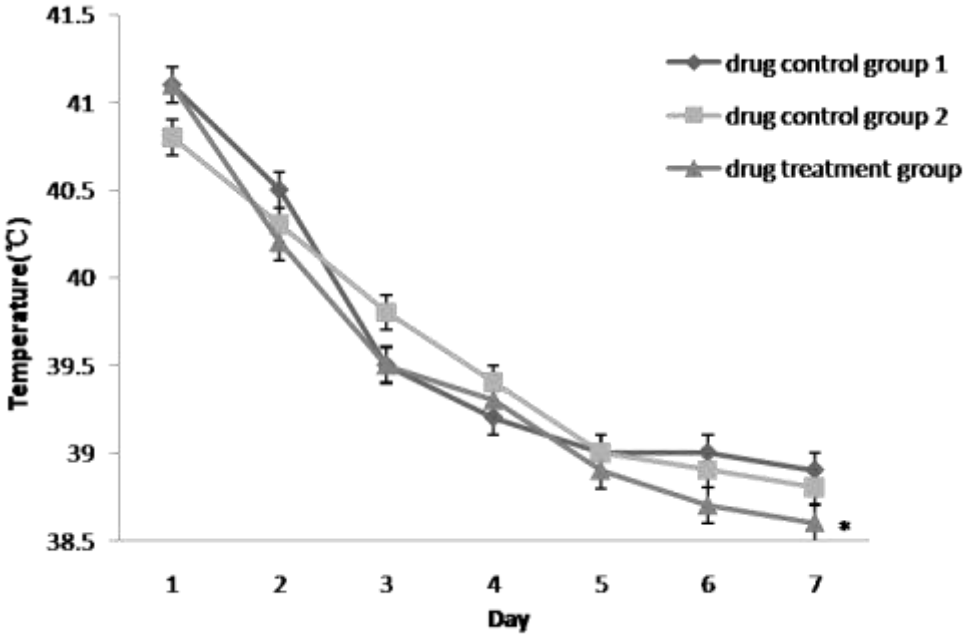

Figure 5: Variation in the body temperature of drug control group 1, drug control group 2, and the experimental drug treatment group over 7 days of treatment. The average temperature of drug control group 1 declined from 41.1 °C to 38.9 °C, a 1.2 °C reduction. The average temperature of drug control group 2 declined from 40.8 °C to 38.8 °C, a 2.0 °C reduction. For the experimental drug treatment group, the average body temperature dropped from 41.1 °C to 38.6 °C, a 2.5 °C reduction. *, significant difference.

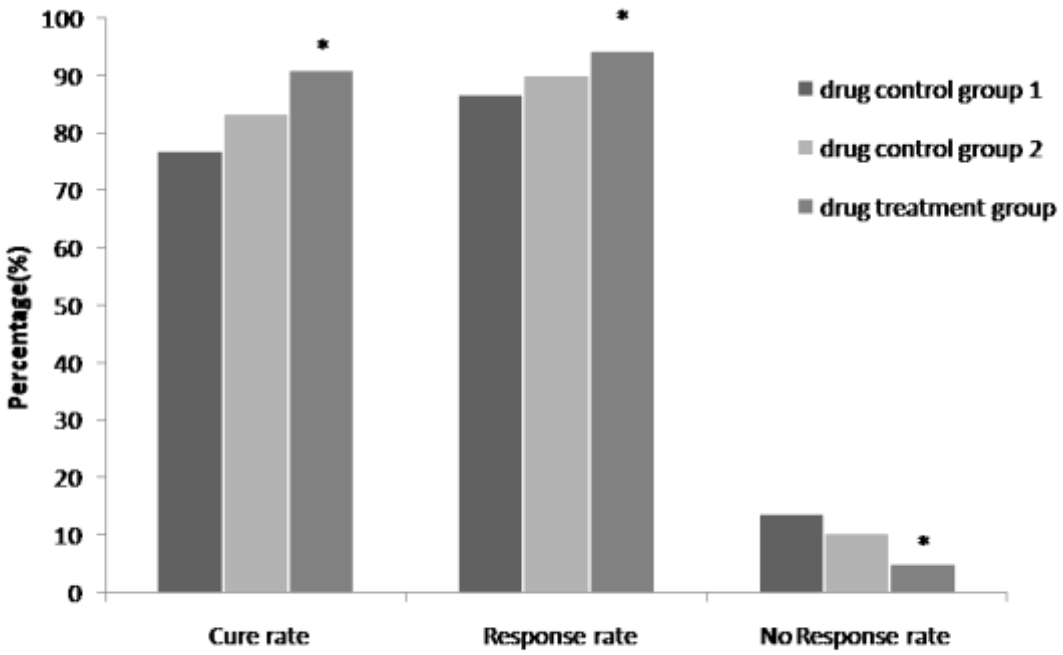

Figure 6: Inhibitory effect of Ban Huang oral liquid against bovine respiratory diseases caused by a mixed infection. The number of ineffective cases was defined as the number of cattle showing no alleviation of symptoms at the end of treatment. Cure rate (%) = (number cured/number affected) × 100. Response rate (%) = (number cured + number of

responsive cattle)/number affected × 100. No response rate (%) = (number of ineffective cattle/number affected) × 100. *, significant difference.

Discussion and Conclusions

Berberine hydrochloride and baicalin were chosen as quality control indices. Other components were analyzed using thin layer chromatography (results not shown). The concentrations of berberine hydrochloride and baicalin in the drug were measured using HPLC. In batch production, the concentrations of berberine hydrochloride and baicalin should be the top priorities of quality control testing if the sources of crude drug are not uniform. The application of the chromatographic fingerprint technique to the evaluation of the quality homogeneity and stability of crude medicinal herbs, semi-finished drugs, and finished drug products is currently under study.

M. bovis infection impairs immunity in the respiratory mucosa of cattle. Most bovine respiratory diseases occur in calves during long-distance transportation. Immunity greatly declines after long-distance transportation, when the cattle are likely to show stress responses and be infected by *M. bovis* and other pathogens. Chinese medicinal herbs destroy the cell membranes and cell walls of pathogens, inhibiting synthesis and expression of proteins. Moreover, TCM herbs may also act directly on DNA structure and interfere with its normal function (Tang et al., 2005), thereby affecting microbial growth and proliferation (Chen et al., 1994), eventually leading to pathogen death (Snyder and Gillies, 2002; Mittra et al., 2000). In this study, the ability of Ban Huang oral liquid to inhibit the growth of *M. bovis* was confirmed in vitro. However, the effectiveness of the drug *in vivo* requires further experimental verification.

Simon Frantz, the chief editor of a column on drug discovery in the journal *Nature*, said that researchers should "forget drugs carefully designed to hit one particular molecule—a better way of treating some complex diseases may be to aim for several targets at once" (Lee et al., 2005). Both humans and animals are complex organisms, which need to be controlled by complex drugs. TCM uses a combination of active components to reach this goal (Kitano, 2007), triggering the immune system and enhancing immunity (Huo and Li, 2002; Shen et al., 2004; Zhang et al., 1998), and thus preventing and treating disease.

Ban Huang oral liquid is easy to prepare, while its quality control methodology is simple. The quality and stability of Ban Huang oral liquid can meet the demands of mass production. Thus, bovine respiratory diseases caused by a mixed infection can be effectively controlled using Ban Huang oral liquid. As a Chinese medicinal product, Ban Huang oral liquid has no toxicity and causes no drug residue or drug resistance problems. Ban Huang oral liquid has great potential for use as a new veterinary drug for respiratory diseases; in addition to therapeutic effects, this preparation has preventive effects against respiratory diseases in livestock and poultry. Because of its good efficacy and safety, Ban Huang oral liquid warrants extensive promotion as a veterinary drug. Moreover, Ban Huang oral liquid provides inspiration for system-oriented drug design and efforts aimed at discovering new drugs to treat and prevent bovine respiratory diseases. Because bovine respiratory diseases have a high rate of incidence and cause significant harm, drug-based prevention is only part of the treatment strategy. All necessary control and prevention measures should be adopted to ensure the healthy development of the animal farming industry by optimizing the breeding and rearing environment, as well as transportation, vaccination, safety isolation, and medication methods.

Acknowledgments

This work was supported by the earmarked fund for the China Agriculture Research System (cars-38) and the Special Fund for Agro-scientific Research in the Public Interest (No. 201303038-4).

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